Highly efficient production of diverse rare ginsenosides using combinatorial biotechnology

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Abstract: The rare ginsenosides are recognized as the functionalized molecules after oral administration of *Panax ginseng* and its products. The sources of rare ginsenosides are extremely limited because of low ginsenoside contents in wild plants, hindering their application in functional foods and drugs. We developed an effective combinatorial biotechnology approach including tissue culture, immobilization, and hydrolyzation methods. Rh2 and nine other rare ginsenosides were produced by MeJA-induced culture of adventitious roots in a 10 L bioreactor associated with enzymatic hydrolysis using six β-glycosidases and their combination with yields ranging from 5.54-32.66 mg L^{-1}. The yield of Rh2 was furthermore increased 7% by using immobilized BglPm and Bgp1 in optimized pH and temperature condition, with the highest yield reaching 51.17 mg L^{-1} (17.06% of PPD-type ginsenosides mixture). Our combinatorial biotechnology method provides a highly efficient approach to acquiring diverse rare ginsenosides, replacing direct extraction from *Panax* plants, and can also be used to supplement yeast cell factories.

Keywords: adventitious roots of *Panax ginseng*, tissue culture, biotransformation of ginsenosides, rare ginsenosides, enzyme immobilization

1 Introduction

The root of *Panax ginseng* C.A. Meyer has been commonly used as a traditional medicine in Northeast Asian countries such as China, Korea, Japan, and Vietnam for more than 2000 years and has become popular in the West over the past few decades (Song, Kim, Choi & Im, 2017; Vo et al., 2015). The major pharmacologically active compounds in ginseng are known as ginsenosides (Kim, Zhang & Yang, 2015), which have various desirable activities, including anti-cancer, antitumor, anti-fatigue, anti-inflammatory, and anti-diabetic effects (Kim & Park, 2011; Riaz et al., 2019; Yu et al., 2017). More than 180 different ginsenosides have been identified, and they can be categorized as oleanane and dammarane type saponins; the latter group includes protopanaxadiol (PPD) and protopanaxatriol (PPT) (Kim, 2012).

The dammarane type ginsenosides Rb1, Rb2, Re, and Rg1, which account for a significant portion of saponins found in ginseng species, are known as major ginsenosides. The PPD-type rare ginsenosides, including F1, F2, Rg3, Rh2, compound Y (CY), compound Mc (CMc), and compound K (CK), have fewer sugar moieties at the C-3 or C-20 positions than major ginsenosides, and can be produced by hydrolysis of the sugar moieties from the major ginsenosides (Park, Yoo, Noh & Oh, 2010). An assay of structure-activity relationships indicated that rare monosaccharide glycosides result in lower viabilities of cancer cells than Rb1 and other major ginsenosides (Quan et al., 2015). For example, Rh2 was shown to significantly inhibit the proliferation of human cancer cells and provoke apoptosis (Li et al., 2011; Oh et al., 1999). BST204, a dry, purified ginseng extract containing a high concentration of racemic Rh2 and Rg3, is being developed for use in supportive care of cancer patients in Korea (Bae et al., 2014). CK has been identified as a main functional component, which has bioactivities of anti-inflammation, hepatoprotection, and anti-diabetes, in addition to anti-cancer effects (Yan et al., 2014). CK was approved by the China Food and Drug Administration for

clinical trials (CDEL20130379) of arthritis prevention and treatment (Yan et al., 2014; Yang, Yang, Ouyang & Yang, 2015).

Despite the versatile bioactivities of rare ginsenosides, their contents in ginseng are extremely low or undetectable in total ginsenosides. To obtain these rare saponins, researchers developed a variety of methods including heterologous synthesis and hydrolysis of major ginsenosides. The yields of rare ginsenosides by heterologous synthesis ranged from about 0.3-2 g L^{-1} in a 5 or 10 L fermenter (Li et al., 2019; Wang et al., 2015; Wang et al., 2019; Zhuang et al., 2017). Although heterologous biosynthesis of ginsenosides by yeast displays tremendous potential to replace phytoextraction, there are still considerable disadvantages. The heterologously expressed enzymes always exhibit lower activity *in vivo*, which is a bottleneck for engineering an efficient cell factory (Sun et al., 2019). Heterogeneous pathways may interact with the native metabolic pathways and disrupt endogenous metabolic flux, which affects the growth and proliferation of the host.

Beside biosynthesis, rare ginsenosides could be obtained through transforming major ginsenosides into rare ginsenosides by acid hydrolysis, alkaline hydrolysis, heating, microbial treatment, or enzymatic transformation (Cui, Wu, Zhao & Yin, 2016; Hong et al., 2012; Li et al., 2019; Park et al., 2017; Song, Kim, Choi & Im, 2017; Sun et al., 2019; Wang et al., 2015; Wang et al., 2019; Yang, Yang, Ouyang & Yang, 2015; Yu et al., 2017; Zhuang et al., 2017). The hot water reflux extraction of total ginsenosides from leaves and roots of *Panax quinguefolium* generates relatively higher contents of Rh2 (11.3 ± 0.5 mg g^{-1}) and Rg3 (10.6± 0.4 mg g^{-1}) (Popovich & Kitts, 2004). Rh2 and Rg3 can also be transformed from a major ginsenoside by mild acidic conditions (Han et al., 1982). But these chemical methods require very rigorous conditions (Bae, Han, Kim & Kim, 2004), and are also associated with some disadvantages, including poor selectivity, epimerization, hydration, hydroxylation, and environmental pollution.

To avoid the disadvantage of chemical methods, the enzymatic biotransformation of ginsenosides was developed to hydrolyze the sugar moiety of ginsenosides to produce rare saponins (Zheng et al., 2017). The major PPD-type ginsenoside Rb1 was hydrolyzed into ginsenoside Rd by removing the outer glucose at the C-20 position. Subsequently, Rd could be converted into Rh2 either via intermediate Rg3 by sequentially cleaving the C-20 inner glucose moiety and the C-3 outer glucose moiety, or via intermediate F2 by cleaving in reverse order (Fig. 1). Recently, many hydrolases with site specific hydrolase activity were discovered, and with the combination of different hydrolases different target minor ginsenosides products can be achieve. The β-glucosidase Bgp1, which specifically hydrolyzes the glycosides of C-20 outer and inner positions, was characterized from *Microbacterium esteraromaticum* and applied to convert Rb1 and Rg3 into Rh2 (Quan et al., 2012). The β-glucosidase from *Gordonia terrae* (Shin, Lee & Oh, 2015), *Paecilomyces* Bainier sp. 229 (Yan et al., 2008), and BglBX10 (Kim et al., 2013) were reported to convert ginsenoside Rb1 to Rg3. The thermostable β-glucosidase Tpebgl3 (Xie et al., 2015) and BGL3T (Pei et al., 2015) were used to convert ginsenoside Rb1 to ginsenoside Rg3. The β-glucosidase BglPm, which specifically hydrolyzes the glycosides of C-3 outer and C-20 outer and inner positions, were characterized from *Paenibacillus mucilaginosus* and recombinantly expressed to convert ginsenosides Rb1 and Rd into F2 (Cui, Kim, Kim & Im, 2014).

The production of CK has been achieved via microbial methods including the use of crude enzymes from *Fusarium sacchari* (Han et al., 2007), *Acremonium strictum* (Chen et al., 2008), and *Lactobacillus paralimentarius* (Quan et al., 2013). To increase the production of CK, the α-L-arabinofuranoside hydrolyzing α-L-arabinofuranosidase (CS-abf) and/or the α-L-arabinopyranoside hydrolyzing β-galactosidase from *Caldicellulosiruptor saccharolyticus* (CS-bgal) were mixed with the β-D-glucopyranoside-hydrolyzing β-glucosidase from *Sulfolobus acidocaldarius* (SA-bglu)

(Shin, Oh, Kim & Oh, 2013). Due to different optimum pH values and temperatures required by each β-glucosidase, few studies have used a combinatorial strategy, such as co-expression of two β-glucosidases to convert major ginsenosides into rare ones, particularly at an industrial production level.

In a previous study, the source of raw material for hydrolyzing major ginsenosides was wild or cultivated ginseng in the field, which requires 5-7 years from its initial planting to harvesting. The yields and qualities are affected by environmental factors, such as soil, climate, shade, pathogens, and pests (Murthy et al., 2014). Tissue cultures offer a number of advantages over the conventional use of plants as sources of phytochemicals: they are independent of geographical, seasonal, and environmental variations; they are reliable in terms of continuous production of uniform quality and yield; they prevent the use of pesticides and herbicides; and they have comparatively short growth cycles (Murthy, Dandin, Park & Paek, 2018). Pneumatically agitated airlift bioreactors are commercially successful for adventitious root and cell suspension culture because of low shear stress, easy to scale-up, low operating and maintenance cost (Baque et al., 2012; Lee et al., 2017; Paek, Murthy, Hahn & Zhong, 2009). Choi et al. (Choi et al., 2000) reported a pilot study on scaling up cultures of ginseng adventitious roots using a 5 to 500 L bioreactor, and showed that the ginsenoside profiles of these multiple adventitious roots were similar to profiles of field-grown ginseng roots. In our previous report (CN 102898493 A) (Li et al., 2011), we designed a balloon type bubble bioreactor for adventitious root production. In a 5 L bioreactor, 15 g ginseng adventitious root was inoculated and harvested after 7 weeks of culture, when the accumulation of major ginsenosides in the ginseng adventitious root was the highest.

In this study, an effective transformation method was developed to obtain rare ginsenosides with a combinatorial approach using culture of ginseng adventitious roots tissue, which have solved the source of total saponins substrate for enzymatic reactions,

combined glycosidase hydrolysis, and enzymatic immobilization technology. Moreover, immobilization of glycosidases was stable and had satisfactory reusability for industrial applications.

2 Methods

2.1 Plant materials and reagents

The 5-year-old *Panax ginseng* C. A. Meyer was obtained from JiAn (Jilin, China). Ginsenoside standards including Rb1, Rb2, Rc, Rd, F2, Rg3, and Rh2 (> 98.0% purity), were purchased from Beijing Solarbio Sciences & Technology Co., Ltd. (Beijing, China). UPLC grade methanol and acetonitrile were purchased from Merck (USA). MeJA (methyl jasmonate), IBA (indole-3-butytric acid), and pNPG (*ρ*-nitrophenyl-β-D-glucopyranoside) were purchased from Sigma-Aldrich (USA). The hollow fiber column (UFP-30-C-2U) used for immobilization was purchased from GE Healthcare (USA).

2.2 Adventitious root culture

The adventitious roots of *Panax ginseng* C. A. Meyer were sterilized and cultured as described by Hahn et al (Hahn et al., 2003). with some modifications. Briefly, the root pieces were inoculated on induction medium containing 1.0 mg L^{-1} 2,4-D, 0.1 mg L^{-1} kinetin, and 30 g L^{-1} sucrose to induce calluses, which were proliferated on MS medium containing 5.0 mg L^{-1} IBA. The adventitious roots were induced from the callus after 4 weeks of culture on MS solid medium containing 3.0 mg L^{-1} IBA and 30 g L^{-1} sucrose in the dark. Approximately 30 g of ginseng adventitious roots were cut into 0.5-1.5 cm pieces then inoculated into 100 mL of liquid MS medium supplemented with 5.0 mg L^{-1} IBA and 30 g L^{-1} sucrose on a rotary shaker (100 rpm) for 7 days. Finally, all the adventitious roots were harvested and inoculated into a 10 L bioreactor with 6 L 1/2 MS medium containing 5 mg L^{-1} IBA and 30 g L^{-1} sucrose. After 7 weeks of initial cultivation, MeJA was supplied to a final concentration of 200 μM for an additional week, then all cultures were rinsed, dried, and submitted to metabolite extraction.

176 **2.3 PPDGM extraction and UPLC analysis**

177 Extraction of protopanaxadiol-type ginsenoside mixture (PPDGM) from the ginseng
178 adventitious roots was performed as described by Shin et al. (Shin, Lee & Oh, 2015) with
179 modifications. In brief, 30 mL of a methanol/water mixture (4:1, v/v) was added to 5 g of
180 dry ginseng root powder and maintained at 37°C overnight. The slurries were extracted
181 three times using 80% methanol for 0.5 h in a sonicator (PL-S40T, KSJ, China) at 240 W
182 at room temperature. The supernatants of each extraction were mixed together, the mixture
183 was filtrated through a 0.45-μm filter, the methanol was removed by evaporation, and the
184 residue was dissolved in 1 mL of distilled water.

185 The ginsenosides were separated and identified via UPLC using an ACQUITY UPLC
186 BEH C18 column (2.1 mm × 50 mm, 1.7 μm) (Waters, USA) in the Acquity UPLC system
187 equipped with an ultraviolet-visible detector (UV-Vis). For the mobile phase, A (water
188 containing 0.05% phosphoric acid) and B (acetonitrile) were used. The gradient elution
189 started with 82% solvent A and 18% solvent B for 5 min, and the flow rate was 0.3 mL
190 min^{-1}. Elution solvents were then changed to 20% B for 1 min and held for 2 min with a
191 flow rate of 0.2 mL min^{-1}, then to 25% B for 1 min and held for 4 min with a flow rate of
192 0.3 mL min^{-1}, followed by 30% B for 4 min and held for 5 min with a flow rate of 0.2 mL
193 min^{-1}, then 40% B for 2 min, and 90% B for 13 min and held for 2 min. Lastly, eluting
194 solvents were changed to 18% B for 1 min with a flow rate of 0.3 mL min^{-1}. The detection
195 was set at 203 nm with an injected volume of 5 μL.

196 **2.4 Recombinant β-glycosidases preparation**

197 Recombinant glycosidases BglPm (Cui, Kim, Kim & Im, 2014), BglSk (Kim et al.,
198 2012), Bgp1 (Quan et al., 2012), BglBX10 (Kim et al., 2013), Tpebgl3 (Xie et al., 2015),
199 and Abf22-3 (Liu et al., 2013) with His-tags were prepared by protein purification from *E.*
200 *coli*. The CDS sequences of *BglPm*, *BglSk, Bgp1, BglBX10, Tpebgl3*, and *Abf22-3*
201 encoding glycosidases and all the primers used in this study were synthesized by

GENEWIZ Technologies Co., Ltd. (Suzhou, China). The DNA fragments were ligated into the *Nde* I/*Xma*l I and *Kpn* I/*Bam*H I sites of a pET14b vector (Novagen) to generate His-Bgp1, His-BglSk, and His-BglPm gene fusions, and pCold-SUMO vector to generate His-SUMO-BglBX10, His-SUMO-Tpebgl3, and His-SUMO-Abf22-3 gene fusions. These vectors were transformed into *E. coli* BL21 (DE3).

The *E. coli* harboring the recombinant plasmid was grown in ampicillin-resistant LB medium at 37°C until the optical density ($OD_{600\ nm}$) reached 0.6. IPTG was added to a final concentration of 0.1 mM for 24 h at 15°C for induction of the fusion protein, then harvested by centrifugation at 3,000 *g* for 30 min at 4°C. The cells were washed with a solution consisting of 20 mM Tris-HCl, 0.5 M NaCl, 30 mM imidazole (pH 8.0), then resuspended in the same solution followed by sonication, and the intact cells and debris were removed by centrifugation at 3,000 *g* for 30 min at 4°C. The His-tagged fusion protein was purified using a Histrap FF affinity column (GE) with AKTA purifier (GE Healthcare, USA). The recombinant proteins were eluted with a solution consisting of 20 mM Tris-HCl, 0.5M NaCl, and 300 mM imidazole (pH 8.0). Finally, the recombinant β-glycosidases were desalted to a solution of 50 mM sodium phosphate buffer (pH 7.5). The protein was assessed by 10% SDS-PAGE followed by Coomassie blue staining. Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay kit (PA115, TIANGEN, China), with bovine serum albumin as the standard.

2.5 Determination of optimum temperature and pH of recombinant β-glycosidases

The optimal hydrolyzing activity of recombinant β-glycosidases at different combinations of temperatures and pHs, in parallel, was assayed as previously described with some modifications (Herlet et al., 2017). Briefly, the reactions consisted of 150 μL sodium phosphate buffer (50 mM) at different pHs (pH 4.5, 5.5, 6.5, 7.5, 8.5, and 9.5) and 10 μL of pNPG substrate solution (5 mM). The reaction mixtures were incubated for 30 min at different temperatures (30, 35, 40, 45, 50, and 55°C) in a gradient PCR cycler

(BioRad, USA). The reaction was stopped by dipping the plate into an ice bath and adding Na_2CO_3 (0.1 M at final concentration). Then, 200 μL of the supernatants were transferred into a 96-well flat-bottom plate and the absorption at 400 nm was determined via a Microplate Spectrophotometer (Tecan, Switzerland). The contour plots were produced by R v3.5.2 function filled.contour() (R Development Core Team 2011). All reactions were performed in triplicate.

2.6 Enzyme reaction kinetics

Kinetic studies were performed with freshly purified enzymes (1-1000 μg mL^{-1}) using pNPG as a substrate at concentrations from 0.1 mM to 10.0 mM, or Rb1, Rd, F2, and Rg3 as substrates at concentrations from 0.2 mM to 5.0 mM. The absorbance of *p*-nitrophenol at 400 nm was monitored for 20 min at pH 8.0 and 25°C. The kinetic parameters, Michaelis-Menten constant (K_m), maximum reaction rates (V_{max}), and catalytic constant (k_{cat}), were determined using the enzyme kinetics program reported by Cleland (Cleland, 1979). According to the Lineweaver-Burk equation, K_m and V_{max} were determined by fitting non-linear regression models to the activities measured at the substrate concentration vs the initial velocity of each reaction. The k_{cat} of Bgp1 and BglPm were determined by plotting the enzyme concentration vs the initial velocity of each reaction. All reactions were performed in triplicate.

2.7 Enzyme hydrolysis on PPDGM

The purified enzyme solutions at concentrations of 0.1 mg mL^{-1} reacted with 3.0 g PPDGM in 50 mM of sodium phosphate buffer (pH 8.0) at 37°C for 24 h. The following experimental groups were included: (1) BglSk, (2) BglPm, (3) Bgp1, (4) BglBX10, (5) Tpebgl3, (6) Abf22-3, (7) BglPm + Bgp1, (8) BglPm + BglBX10, (9) BglPm + Tpebgl3, (10) BglPm + Abf22-3, (11) BglSk + Bgp1, (12) BglSk + BglBX10, (13) BglSk + Tpebgl3, (14) BglSk + Abf22-3, and (15) BglSk + BglPm. Then an equal volume of water-saturated *n*-butanol was added to stop the reaction, and the reactant present in the *n*-butanol fraction

was evaporated in a water bath at 60°C, then dissolved in methanol and filtered with 0.45 μm filter membrane. The final filtrate was subject to the UPLC analyses. The conversion yield (%) was presented as Δ product / (Σ Δ substrate) × 100. All reactions were performed three times.

2.8 Storage stability of the immobilized enzyme

The polysulfone hollow fiber membrane with 30 kDa molecular weight cut-off (MWCO) was used for immobilizing enzymes. The membranes were equilibrated in phosphate buffer (pH 8.0) for 30 min. A pressure-driven filtration process was carried out for enzyme immobilization. The enzyme solution (0.5 mg mL^{-1}) was pumped into the lumen flowing through the membrane under a recirculation loop for 2 h using a peristaltic pump with a 10 mL min^{-1} flow rate. The enzymes were entrapped in the asymmetric, aqueous-filled ultraporous membrane and retained by the 30 kDa skin on the inner surface (Fig. 6). The amount of immobilized biocatalyst was measured by mass balance between the initial solution and the collected fractions at different time points (10, 20, 30, 40, 50, 60, 70, 80, 90, and 120 min) (Morthensen, Meyer, Jørgensen & Pinelo, 2017).

The storage stability of the immobilized β-glycosidases was monitored for 15 days at 25°C by carrying out the hydrolysis of pNPG under a standard assay. The reaction mixture contained 50 mL of 50 mM sodium phosphate buffer at pH 8.0, 0.5 mM pNPG, and 50 mg immobilized β-glycosidases.

For running each cycle, 5 mL of pNPG substrate solution (5 mM) was added into the feed tank then pumped to circulate in the membrane reactor at the maximum flow rate (5 mL min^{-1}) for 0.5 h at 25°C. The absorption of the supernatants at 400 nm was detected to determine the concentration of *p*-nitrophenol. The activity of the immobilized enzyme after the first cycle was defined as the control and assigned a relative activity of 100%. The residual activity was calculated by dividing the enzyme activity on that particular day by the enzyme activity at the start of the storage stability experiment (on day 1) (Palai,

Singh & Bhattacharya, 2014). The assays were performed in triplicate.

2.9 Production of Rh2 from continuous hydrolyzing of PPDGM by the non-immobilized and immobilized β-glycosidases

For the non-immobilized β-glycosidase hydrolysis reaction, 30 mg mL^{-1} of PPDGM and non-immobilized purified enzymes (1.0 mg mL^{-1}) were incubated in 100 mL of 50 mM sodium phosphate buffer (pH 8.0) at 25°C for 6 h, 12 h, 24 h, and 48 h. In total, 100 μL of the supernatants were collected and extracted using methanol for UPLC detection.

For the immobilized β-glycosidase hydrolysis reaction, the schematic representation of the experimental set up is shown in Fig. 6. The biocatalytic membrane reactor with immobilized BglPm and/or Bgp1 (50 mg each) was set up as described in the section on the storage stability test of the immobilized enzyme. Then, 100 mL PPDGM solution (30 mg mL^{-1}) was added to the feed tank and pumped to circulate the membrane reactor with the maximum flow rate (5 mL min^{-1}) for 48 h at 25°C. A total of 100 μL of the supernatants were collected and purified using methanol for UPLC detection at 6 h, 12 h, 24 h, and 48 h.

3 Results

3.1 Efficient production of PPD-type ginsenosides mixture (PPDGM) by adventitious roots culture in a bioreactor

The bioreactor culture of ginseng adventitious roots is a scaled-up culture with three steps (Figs 2A-D). Adventitious roots were first propagated for 30 days on a solid proliferation medium at 25°C in dark conditions using a few root fragments as an initial inoculation (Figs 2A, B). Then, about 30 g of cultured adventitious roots was collected and transferred to the liquid culture in a gyratory shaker (Kim et al., 2013) for 7 days (Fig. 2C). After the ginseng root was transplanted from the shaker to the bioreactor, the biomass of the ginseng adventitious root did not increase within the first 4 weeks. From the 5^{th} week to the 7^{th} week, the ginseng roots were in the rapid growing period, and grew slower

after entering the 8th week. Therefore, 200 μM MeJA was added on the 50th day to induce an increase in the ginsenoside content, and 785.98 ± 29.43 g fresh roots were harvested for metabolite extractions on the 56th day (Fig. 2D).

The PPDGM yielded from 10 L culture under MeJA induction was 0.30 ± 0.02 g L^{-1}, 3-fold higher than that from control culture (0.10 ± 0.01 g L^{-1}). Ultra-high performance liquid chromatography (UPLC) analysis of major protopanaxadiol-type ginsenosides (Rg1, Re, Rb1, Rd, Rc, and Rb2) and one rare ginsenoside (F2) indicated that the contents of all monomeric ginsenosides in MeJA-induced adventitious roots was significantly higher than that of uninduced roots, but similar to or lower than that of 5-year-old wild *Panax ginseng* roots (Fig. 2E). In detail, the content of Rb1 increased from 0.27 ± 0.14 mg g^{-1} dry weight (DW) of ginseng root to 1.75 ± 0.01 mg g^{-1}; and the content of Rc increased from 0.05 ± 0.01 mg g^{-1} DW of ginseng root to 1.12 ± 0.11 mg g^{-1}. The content of Rd in the adventitious roots was 1.29 ± 0.37 mg g^{-1}, as high as that in 5-year-old wild ginseng roots (1.49 ± 0.19 mg g^{-1}). The content of Rb2 in the adventitious roots was 0.61 ± 0.06 mg g^{-1}, one third of that in 5-year-old wild ginseng roots (1.88 ± 0.27 mg g^{-1}) (Fig. 2E). The content of rare ginsenoside F2 in the adventitious roots was only 0.20 ± 0.01 mg g^{-1}, similar to that in 5-year-old wild ginseng roots. In summary, major ginsenosides were successfully extracted from culture of adventitious roots in a 10-L bioreactor after 2 months, which could partially replace that from five years of growth of wild plants.

3.2 Selection, purification, and characterization of β-glycosidases for PPDGM hydrolysis

To increase the content of rare ginsenosides in PPDGM, enzymatic transformation was used to produce rare ginsenosides. Six β-glycosidases that can specifically hydrolyze the C-3 and C-20 sugar moieties of ginsenosides were selected to produce rare ginsenosides by enzymatic catalysis. To hydrolyze the glycoside moieties of C-3 of Rb1, Rd, Rc, and Rb2, BglSk from *Sanguibacter keddieii* and BglPm from *Paenibacillus mucilaginosus*

were selected based on activities reported in previous studies. Three other β-glycosidases (Bgpl, BglBX10, and Tpebgl3) from *Microbacterium esteraromaticum*, *Flavobacterium johnsoniae*, and *Thermotoga petrophila* were respectively chosen to hydrolyze C-20 sugar moieties of the above major ginsenosides. To hydrolyze α-L-arabinofuranoside of Rc, Abf22-3 from *Leuconostoc* sp. 22-3 was also used in our screening experiments.

The glucosidase genes *bglSk*, *bgp1*, and *bglPm* were synthesized and amplified via PCR and then inserted into the pET14b vector, and *bglBX10*, *tpebgl3*, and *abf22-3* were cloned into the pCold-SUMO vector. Compared to the uninduced case, an induction with 0.1 mM IPTG at 15°C for 24 h cultivation after induction produced the maximum level of fusion protein of Bgp1, BglSk, BglPm, BglBX10, Tpebgl3, and Abf22-3 in the supernatant from cell lysates. SDS-PAGE analysis of Ni^{2+}-binding agarose resin purified proteins showed a single main band of 95 kDa for His-Bgp1, 48 kDa for His-BglPm, 71 kDa for His-BglSk, 105 kDa for His-SUMO-BglBX10, 96 kDa for His-SUMO-Tpebgl3, and 70 kDa for His-SUMO-Abf22-3 (Fig. 3A). Those results indicated that recombinant proteins were successfully highly expressed in soluble form, increasing possibility of industrial applications.

To characterize optimal pH and temperature for activities of recombinant β-glucosidases, the hydrolysis activity was studied using pNPG as a substrate in the buffers at various pH values (4.5-9.5) from 30-55°C. BglPm had broad optimum conditions under all investigated temperatures at pH values between 7.8 and 9.5 (Fig. 3B). Optimization of BglSk needed similar pH conditions as BglPm, but the temperature was constrained at 30-40°C (Fig. 3B). Bgp1, BglBX10, and Abf22-3 had more than 80% activity at similar pH and temperature conditions: pH between 7.5 to 8.5 and temperature at 30 to 37°C (Fig. 3B). In contrast, Tpebgl3 had higher activity under a high temperature condition from 37 to 55°C (Fig. 3B). In summary, most β-glucosidases have optimal pH and temperature at pH 8.0 and 37°C, respectively, which were applied in the following

ginsenosides conversion.

3.3 Producing diverse rare ginsenosides by β-glycosidases hydrolysis

Fifteen enzymatic transformations including six single enzyme and nine different combinatorial enzyme treatments were carried out using PPDGM as a substrate in 50 mM sodium phosphate buffer (pH 8.0) at 37°C for 24 h. The relative contents of 15 detectable ginsenosides of enzymatic reactions as well as untreated control were quantified by UPLC analysis (Fig. 3C). As shown in supplementary data, four major ginsenosides – Rb1, Rc, Rb2, and Rd – accounted for 22.95 ± 1.08%, 24.26 ± 0.87%, 20.10 ± 0.93%, and 15.68 ± 0.47% of untreated PPDGM, respectively (Fig. 3D). Single enzymatic treatment by BglPm produced a large amount of F2 (262.83 ± 4.92 mg) accounting for 46.12 ± 1.39% of PPDGM, as well as 13.72 ± 0.42% and 13.39 ± 0.40% of CMc1 and CO, respectively (Figs 3D, E). BglSk can also hydrolyze all major ginsenosides and produced Gyp75, CMc, CY, and CK at proportions higher than 20% (Fig. 3D). Bgpl, BglBX10, and Abf22-3 converted Rd into Rg3, and Tpebgl3 transformed both Rb1 and Rd into Rg3 (Figs 1, 3D).

The production of Rg3 in the above four reactions accounted for about 15% to 30% of PPDGM, peaking at 149.74 ± 5.67 mg by Tpebgl3 (Fig. 3E). All combinatorial enzymatic treatments of BglPm with four other enzymes with C-20 hydrolysis activities (Bgpl, BglBX10, Tpebgl3, and Abf22-3) produced more than 40% of Rh2, and more than 10% of CMc1 and CO (Fig. 3D). BglSk combined with these four enzymes produced a moderate amount of Rg3, Gyp75, CMc, CY, and Rh2 with proportions between 10% and 20% (Fig. 3D). A high proportion of CK (277.61 ± 4.27 mg, 34.22 ± 1.91%) was produced in the treatment by both BglPm and BglSk (Figs 3D, E). Among all transformations, BglPm+Bgp1 hydrolyzed 3.0 g PPDGM and produced the highest yields of Rh2 (326.61 ± 7.04 mg), accounting for 10.89 ± 0.23% of PPDGM (Fig. 3E).

3.4 Increasing rare ginsenosides production efficiency by multienzyme immobilized polymeric membrane reactor

To facilitate the usage efficiency of enzymes and separation between enzymes and products, Rh2 transformation using recombinant His-Bgp1 and His-BglPm immobilized on the hollow fiber membrane were carried out as an example to validate our strategy. A previous study showed that activities of β-glucosidase were stable from 5 to 30°C, were gradually reduced between 30 to 45°C, and were significantly reduced above 45°C (Cui, Kim, Kim & Im, 2014). For stabilizing enzyme activities, further ginsenosides conversion reactions based on enzymatic immobilization were carried out at pH 8.0 and 25°C.

The efficiency of immobilization was determined by determining initial protein and subtracting protein remaining in solution vs. time of incubation with each enzyme alone or their combinations. There was a rapidly increased level of immobilized enzymes during the first 60 mins, followed by a slow increase over the next 30 mins (Fig. 4A). After 2 hours of loading, the recombinant enzymes on the hollow fiber membrane reached a stable level at about 45 mg, which indicated that the porous region (~50 cm^2) of an asymmetric hollow fiber membrane was fully loaded at the final concentration of 0.9 mg cm^{-2} (Fig. 4A).

As the reusability of immobilized enzymes is the most important feature for large-scale industrial applications, we studied the storage stability of the immobilized or free enzymes within 15 days at room temperature (25°C) (Fig. 4B). The immobilized β-glucosidases showed high recycling efficiency, retaining about 85% of their relative activity after nine cycles of catalytic reaction on the ninth day. The immobilized β-glucosidases were still capable of remaining at 40% of the initial activity even after 15 cycles of successive catalytic reactions (Fig. 4B).

The kinetics of both free and immobilized enzymes were studied by measuring the reaction rates with two different substrates: pNPG and ginsenosides. The K_m values for Bgpl and BglPm were 0.85 ± 0.21 mM and 0.40 ± 0.12 mM, respectively, significantly higher than those for ginsenosides, indicating a high affinity for their natural substrates. In

particular, the K_m value of BglPm for Rb1 was 0.04 ± 0.0003 mM, an order of magnitude lower (Table 1). We also measured V_{max} and calculated k_{cat} for the recombinant His-Bgp1 and His-BglPm for pNPG. Catalytic efficiency (k_{cat}/K_m) depended on both enzyme and substrate, with remarkably high values for BglPm with pNPG (154.08 $s^{-1}mM^{-1}$), indicating high activity performance, and low values for Bgp1 with Rb1 (1.88 $s^{-1}mM^{-1}$), showing the low efficiency of this biocatalyst (Table 1). The V_{max} value for the immobilized enzymes was about 5-fold higher than the apparent level of free enzyme, suggesting an elevated level of activities of hydrolysis (Table 1). The K_m of free and immobilized enzymes was at a similar level, indicating that immobilization did not change the affinities for the substrate (Table 1).

The transformation efficiency was investigated by analyzing increased contents of major and rare ginsenosides at different times after 3.0 g PPDGM was added into the reaction pools with immobilized Bgp1 and BglPm, or their free counterparts (Fig. 5). Rb1 and Rd were consumed within 12 hours, and the intermediate product Rg3 was accumulated in the beginning reaction followed by a gradual decrease (Fig. 5). Other intermediate products including F2, Gyp17, Gyp75, and CK remained at a stable low level during the whole reaction process (Fig. 5). In the free enzymes system, Rh2 was accumulated to 300.30 ± 28.26 mg in 24 hours, accounting for 10.01% of PPDGM. Rh2 was accumulated to 511.72 ± 3.04 mg in the immobilization system, accounting for 17.06% of PPDGM (Fig. 5). The final product of ginsenosides PPD was gradually increased during the process of hydrolysis (Fig. 5). Immobilized glucosidase could facilitate enzyme recycling in sequential glycosyl hydrolysis processes to produce rare ginsenosides, thereby reducing enzyme cost. The conversion yield of Rh2 was 68.32% for the two combined glucosidases.

4 Discussion

This work provided a set of solutions including culture of ginseng adventitious roots,

hydrolysis of major ginsenosides, and the immobilization of β-glucosidases, producing large amounts of ten types of rare ginsenosides (Fig. 6). In a previous study (Song, Kim, Choi & Im, 2017), 2.27 g Rh2 were obtained from 60 g PPDGM with the yield of 37.8 g kg^{-1} using the commercial enzyme Viscozyme L followed by acid treatment. We produced 511.72 mg Rh2 from 3.0 g PPDGM with the yield of 170.57 g kg^{-1}. The yield of our method could be further improved as described below.

The substrate specificifity of β-glucosidases are normally determined by the position and type of sugar moieties of the ginsenosides. There are five different types of sugar moieties, including β-D-glucopyranoside, α-L-arabinopyranoside, α-L-arabinofuranoside, β-D-xylopyranoside, and / or α-L-rhamnopyranoside in the ginsenosides. Our strategy thus relies on the four types of β-glucosidases and provides a universal way to produce diverse rare ginsenosides. For Rh2 transformation, Rb2 and Rc were hydrolyzed into CO and CMc1 by BglPm; Rb1, Rb2, and Rc were respectively hydrolyzed into Gyp17→Gyp75, CO→CY, and CMc1→CMc by BglSk (Fig. 1), which were also the side products of enzymatic reactions in previous research (Cui, Kim, Kim & Im, 2014; Kim et al., 2012). In future experiments, the yields of Rh2 will be improved through constructing a multi-enzyme system with additional enzymes, which could hydrolyze α-L-arabinopyranosidase and α-L-arabinofuranosidase of major ginsenosides Rb2 and Rc. Such glycosidases include Bgp2, which has the transformation pathways Rb2→Rd→Rg3, C-O→F2→Rh2 (Quan et al., 2013), and Abf22-3, which can hydrolyze Rc into Rd (Liu et al., 2013). However, Abf22-3 activity was only detected in hydrolyzing β-D-glucopyranoside rather than α-L-arabinofuranosidase in this study.

Moreover, immobilization of glycosidases was stable and had satisfactory reusability for industrial applications. Bgp1 and BglPm with different molecular weights can be immobilized on the membrane at the same time because of the different pore sizes of the membrane. The K_m remained unchanged after immobilization (Table 1), indicating that the

structure of the enzyme had not been destroyed. The V_{max} had a 5-fold increase, indicating that the reaction rate and enzymatic effect of the enzyme were increased after immobilization. In other studies, some increases in K_m have been reported for glucosidases immobilized on other carriers (Torres-Bacete et al., 2000; Tu et al., 2006). Immobilized enzymes normally have lower V_{max} values than their free counterparts (Petzelbauer, Splechtna & Nidetzky, 2002; Saville et al., 2004). But higher V_{max} values following immobilization were reported for β-galactosidases and other glucosidases (Lamb & Stuckey, 2000; Saville et al., 2004; Tu et al., 2006). The mechanistic basis of immobilization impacts on V_{max} is complicated and requires further investigation.

The stability of the enzyme can last about ten days, during which time saponins can be reacted continuously to improve the utilization rate of the enzyme. Most major ginsenosides are converted into rare ginsenosides, which shows that the method and the platform system are feasible, and can be used for the biotransformation of other low-sugar-moiety saponins. Commercial scale-up of high-value chemicals production in a tonnage bioreactor using the proposed biotechnology approach therefore seems feasible and promising (Gai et al., 2017).

Acknowledgements

The authors gratefully acknowledge financial support from the Applied Technology Research and Development Program Major Project of Heilongjiang Province (No. GA16C106), the Fundamental Research Funds for the Central Universities (No. 2572018CP04, No. 2572016BA06 and No.2572014AA19), the National Natural Science Foundation of China (NSFC Grant No. 31770332, 31970314), the Opening Project of Zhejiang Provincial Preponderant and Characteristic Subject of Key University (Traditional Chinese Pharmacology), and the Zhejiang Chinese Medical University (No. ZYAOX2018012), the National Key Research and Development Program of China

(2017YFD0201404). We are especially grateful to Gaobo Liu for technical assistance and illuminating discussions on the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Author contributions

YW, YHL and ZYX designed the experiments. LGC and HW performed the experiments, with help of HZ, QZ, XY, DRZ, CWL, MJK and PK. LGC, HW, YW and ZYX analyzed the data and wrote the manuscript.

Supporting information

Table S1. The peak area percentage of PPD-type saponin detected after glycosidases conversion of PPDGM.

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685

686 **Figure legends**

687 **Fig. 1 The proposed biotransformation pathways of PPD type ginsenosides by**
688 **glycosidases.**

689 Rb2, Rc, Rb1, and Rd in the grey ring are major ginsenosides; CY, CMc, CMc1, CO,
690 Gyp17, Gyp75, CK, F2, Rh2, and Rg3 in the pink ring are rare ginsenosides. Ap:
691 arabinopyranoside; Af: arabinofuranosidse; G: glucopyranoside. The notes on the arrows
692 represent the cleavage position of glycosidic bonds, such as C-3 outer: cleavage of sugar
693 moiety on C-3 position.

694 **Fig. 2 Efficient production of ginsenosides by culture of ginseng adventitious roots in**
695 **a bioreactor.**

696 (**A**) Ginseng adventitious roots were inoculated on solid proliferation medium; (**B**)
697 proliferation of adventitious roots grown on solid medium in 1 month; (**C**) adventitious
698 roots were further cultured in liquid medium for 7 days; (**D**) the adventitious roots were
699 scaled up to a 10 L bioreactor and grown for 2 months. (**E**) Content of major ginsenosides
700 in harvested adventitious roots and 5-year-old wild ginseng roots. Data are expressed as a
701 percentage of ginsenosides of dry weight ginseng root and presented as mean ± SD (n = 3).
702 * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ treated versus untreated control, wild versus treated
703 GARs. GARs: Ginseng adventitious roots.

704 **Fig. 3 Expression, screening, and biotransformation of glycosidases.**

705 (**A**) SDS-PAGE analysis of the purified recombinant glucosidases. Lanes: Marker,
706 molecular weight standard (180, 130, 95, 72, 55, 43 kDa); BglPm, His-BglPm after
707 purification; BglSk, His-BglSk after purification; Bgp1: His-Bgp1 after purification;
708 BglBX10, His-SUMO-BglBX10 after purification; Tpebgl3, His-SUMO-Tpebgl3 after
709 purification; Abf22-3, His-SUMO-Abf22-3 after purification. (**B**) Contour plot of
710 glycosidases BglPm, BglSk, Bgp1, BglBX10, Tpebgl3, and Abf22-3 using pNPG as a
711 substrate to evaluate temperature vs. pH. (**C**) UPLC analysis of transformation via treating

PPDGM with glycosidase combinations for 24 hours. 1: Gyp17; 2: CMc1; 3: CO; 4: Gyp75; 5: Rg3; 6: CMc; 7: CY; (**D**) Heatmap representation of the relative content of PPD-type ginsenosides in glycosidase conversion reactions (data is shown in Table S1); (**E**) The highest yields of each rare ginsenoside among all glycosidase combinations. Note: Gyp17, CMc1, CO, Gyp75, CMc, and CY were relatively quantified using the standard curve of Rh2 due to lack of available standards. F2, Rg3, CK, and Rh2 were absolutely quantified using their own standard curves. Abbreviations: Pm: BglPm; Sk: BglSk; Std, Standards mixed ginsenosides Rb1, Rc, Rb2, Rd, F2, Gyp17, CMc1, CO, Gyp75, CMc, CY, Rg3, Rh2, CK and PPD. PPDGM: protopanaxadiol-type ginsenoside mixture.

Fig. 4 Loading efficiency and thermal stabilities of immobilized β-glucosidase.

(**A**) Bglpm and Bgp1 immobilized on a hollow fiber column; (**B**) storage stability determination of immobilized Bgp1 and BglPm. The amount of immobilized protein was determined from the difference between the total protein added and the amount remaining in solution after immobilization. The activity of the immobilized enzyme was assayed using pNPG as the substrate.

Fig. 5 UPLC results of transformation of PPDGM by β-glucosidases Bgp1 and BglPm combination.

Time course analysis of Rb1, Rd, Rg3, F2, Gyp17, Gyp75, CK, Rh2, and PPD in free and immobilized Bgp1 and BglPm glycosidase combinations. Note: Gyp17 and Gyp75 were relatively quantified using the standard curve of Rh2 due to lack of available standards. Rb1, Rd, PPD, F2, Rg3, CK, and Rh2 were absolutely quantified using their own standard curve.

Fig. 6 Schematic diagram of rare ginsenosides biotransformation system.

Our method includes adventitious roots culture and immobilized β-glycosidases hydrolysis, resulting in diverse rare ginsenosides production at a larger scale. PPDGM: protopanaxadiol-type ginsenoside mixture.

739 **Table 1. The kinetic parameters of recombinant BglPm and Bgp1**

Name	Substrate	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
Bgp1	pNPG	0.85±0.21	8.49 ±0.62	16.83±0.11	19.80±0.13
	Rb1	0.15 ±0.01	1.75 ±0.08	0.28 ± 0.01	1.88 ± 0.08
	Rd	0.25 ± 0.03	16.05 ± 1.09	2.78 ±0.19	11.02 ±0.21
	F2	0.15 ±0.02	8.99 ±0.34	2.04 ± 0.10	13.94 ± 0.09
BglPm	pNPG	0.40±0.12	24.59 ±0.60	61.63±0.61	154.08±1.52
	Rb1	0.04 ±0.003	3.29 ±0.33	0.49 ±0.04	12.63 ± 0.04
	Rd	0.11 ±0.01	9.16 ±0.56	1.58 ±0.21	14.07 ±0.23
	Rg3	0.17 ±0.01	8.38 ±0.47	2.08 ±0.42	11.94 ±0.47
Immobilized Bgp1	pNPG	0.87±0.11	45.78 ±12.54	ND	ND
Immobilized BglPm	pNPG	0.41±0.13	90.24 ±17.12	ND	ND

740 ND: Not determined
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